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Exhibit A

Stress-Induced Changes in Intestinal Transit in the Rat: A Model for Irritable Bowel Syndrome

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Stress in humans commonly results in gastrointestinal dysfunction, which is characterized by its symptomatology because the etiology is completely unknown. We developed an animal model in which to study the effects of stress on the gastrointestinal tract, and characterized the model as a stressor by evaluating endocrine and analgesic responses to mild restraint. Mild restraint (wrap restraint) elevated plasma levels of adrenocorticotropic hormone and β-endorphin, and caused analgesia. The different regions of the gastrointestinal tract responded differently to the stress stimulus. Gastric emptying was not affected, small intestinal transit was inhibited, and large intestinal transit was stimulated by stress, and there was an associated increase in fecal excretion. Wrap-restraint stress did not result in the formation of ulcers. There was a strong correlation between stress-induced adrenocorticotropic hormone release and stress-induced intestinal dysfunction over a 24-h period that suggested a circadian influence. However, neither exogenous adrenocorticotropic hormone nor β-endorphin had any effect on intestinal transit. Furthermore, neither adrenalectomy nor hypophysectomy prevented the response of the intestine to stress, suggesting that neither adrenal nor pituitary-derived factors are responsible for mediating the effects of stress on the gut. We conclude that wrap-restraint stress produces different effects on different regions of the intestine, suggesting that the small and large intestines are independently regulated and can respond differently to different stimuli. There were similarities between the intestinal effects of wrap-restraint stress in rats and intestinal symptoms associated with stress and irritable bowel syndrome in humans. Therefore, wrap restraint may be an appropriate animal model in which to study stress-related intestinal dysfunction. The mechanisms by which stress affects intestinal transit are still unresolved; however, the intestinal effects of stress are not mediated by either pituitary or adrenally derived factors.

The sensitivity of the gastrointestinal tract to stress has been demonstrated in clinical settings for over half a century (1). Stress and peptic ulcers have been linked clinically and in laboratory studies. However, stress-related gastrointestinal dysfunction also includes another major pathology: stress-induced alterations in intestinal motility and transit. A distinct relationship between stress and intestinal motility was first demonstrated by Walter Cannon (2), who noted changes in the contour and flow of intestinal contents in cats confronted by a growling dog. However, the specific actions of stress on intestinal transit have not been well documented. Attempts to elucidate the mechanisms by which stress affects intestinal motor activity have led to conflicting results, possibly because different stress models were used in different studies. The lack of an appropriate animal model has hindered studies of causality. Recently, we reported a comparison of a number of stress models and the resulting range of gastrointestinal responses that were associated with different stressors (3). We found that different stress paradigms initiated different degrees of intestinal dysfunction, and, further, that stress-related intestinal dysfunction was mediated by different neurochemical pathways in different stress models. These studies have two important implications. First, it is clear that stress results in a perturbation of the entire

Abbreviation used in this paper: IBS, irritable bowel syndrome.

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internal milieu of an animal, and that resultant changes seen in a specific end organ such as the gut are very dependent on the type of stress, as well as a number of other factors, such as animal species, strain, gender, age, previous stress experience, and intrinsic biological factors such as the light-dark cycle. Second, to study the effects of stress on the gastrointestinal tract, care must be taken to eliminate as many extrinsic factors as possible, and to evaluate the importance of intrinsic factors in the stress response. The purpose of this study was threefold: (a) to evaluate the effects of a mild form of restraint on gastric emptying and small and large intestinal transit, (b) to determine the reliability of the restraint as a stress stimulus by measuring changes in plasma adrenocorticotropic hormone (ACTH) and β-endorphin concentrations (the pituitary-adrenal axis is the endocrine system most central to the stress response, and pituitary ACTH and β-endorphin are sensitive indicators of the generalized stress response); and (c) to investigate the potential of pituitary or adrenally derived factors as mediators of the effects of stress on the intestine.

Materials and Methods

Animals

Female Sprague–Dawley rats (150–200 g) were used in all experiments. The animals were housed in groups of 5 in a temperature-controlled room on a 12-h light-dark cycle (7 AM to 7 PM) and were allowed water and Rat Chow (Ralston Purina, St. Louis, Mo.) ad libitum. Animals were allowed at least 1 wk to acclimate to the environment before experiments were performed.

Stress

The stress model used in all experiments was "wrap-restraint stress," a novel procedure that is milder than classical restraint models in that it does not result in the formation of ulcers (see Results). We were concerned that mucosal ulcers produced by classical restraint stress models, such as cold restraint, would act as an additional, uncontrolled source of stress and complicate interpretation of our results.

Animals were stressed by partially restraining them in a harness of paper (masking) tape to restrict movement of the upper body and forelimbs. Rats were lightly anesthetized with ether and their foreshoulders, upper forelimbs, and thoracic trunk were wrapped in paper tape to restrict, but not prevent, movement. The animals recovered from ether within 2–5 min and immediately moved about in their cages and ate and drank, but had restricted mobility of their forelimbs, which prevented them from grooming the face, upper head, and neck. Control animals were anesthetized with ether but were not wrapped. After recovering from ether anesthesia, control rats diligently groomed the face, head, and abdomen.

Gastrointestinal Transit

Rats were anesthetized with a mixture of pentobarbital and chloral hydrate [Equithesin (Jensen-Salsbery Laboratories, Kansas City, Mo.) (4)], the abdomen was opened by a midline incision, and a chronic indwelling Silastic cannula (Dow Corning, Midland, Mich.), 0.5 mm ID and 0.9 mm OD, was implanted into the proximal duodenum and proximal colon. The cannulas were inserted into the lumen of the intestine and secured with a suture. The cannulas were brought subcutaneously to the scapular region of the back, where they were externalized and housed in a plastic cylinder (fashioned from the barrel of a 3-ml plastic syringe) sutured to the skin and closed with a cork. Rats were individually housed and allowed to recover from surgery for 3–5 days. All transit experiments were performed in animals that had been fasted for 18–24 h.

Small and large intestinal transit were evaluated in rats by instilling 0.5 μCi of ^{51}Cr, as sodium chromate dissolved in distilled water (0.2 ml), directly into the duodenum or colon via the implanted cannula. Gastric emptying was evaluated by oral administration (gavage 0.2 ml) of the radiomarker. The radioactive chromium served as a nonabsorbable marker for measuring the quantitative movement of contents along the lumen of the bowel. To study the effects of wrap-restraint stress on intestinal transit and gastric emptying, rats were lightly anesthetized with ether, wrapped, and immediately given chromium by oral gavage or via the indwelling luminal cannula. Control animals were briefly anesthetized with ether, handled in a manner to suggest "sham wrapping," and given chromium. This brief exposure to ether did not affect normal transit. Thirty-five minutes after the administration of the radioactive marker, the animals were killed by cervical dislocation and the small and large intestine were removed and each was divided, without spillage of contents, into 10 (small intestine) and five (large intestine) equal segments by use of a premeasured template. In determining large bowel transit, the feces were saved and counted for γ-radiation. The feces represented an additional segment of the large intestine and were included in the calculation of the geometric center for colonic transit. Each segment, and the fecal material, was placed into an individual vial and counted for γ-emissions for 1 min in a γ-counter (Tracor Analytic, Elk Grove Village, Ill.). The amount of radioactivity determined for the individual segments was used to calculate the geometric center of transit, a quantitative evaluation of the distribution of the radioactivity along the small and large intestine (5), according to the following equation:

$$GC = \sum \frac{\text{Counts per segment} \times \text{segment number}}{\text{Total counts}},$$

where GC is the geometric center.

Geometric centers range from values of 1 to 10, such that a geometric center of 1 indicates that transit through the small intestine was maximally inhibited, whereas a geometric center of 10 indicates that transit was maximal. The percent change in intestinal transit was calculated according to the following equations:

$$\% \text{ Inhibition} = \frac{\text{Control GC} - \text{stress GC}}{\text{Control GC} - 1} \times 100,$$

$$\% \text{ Increase} = \frac{\text{Stress GC} - \text{control GC}}{\text{Control GC}} \times 100.$$

To evaluate gastric emptying, the stomach was placed in a test tube, and the intestine, cut into 10 equal segments as for transit evaluation, was put into test tubes, and the total amount of radioactivity in the stomach and small intestine was determined by γ-radiation. The amount of radioactivity emptied from the stomach, expressed as percent of gastric emptying, was calculated according to the following equation:

$$\% \text{ Gastric emptying} = \frac{\text{Total counts} - \text{stomach counts}}{\text{Total counts}} \times 100.$$

All experiments were performed between 4 and 6 PM, with one exception. To study the effects of circadian influences on stress-induced inhibition of intestinal transit, transit was assessed in wrap-restraint-stressed rats and nonstressed control rats at 1 AM, 4 AM, 7 AM, 10 AM, 1 PM, 4 PM, 5 PM, 7 PM, and 10 PM.

Adrenocorticotropic Hormone and β-Endorphin Assays

To determine that our model was indeed a "stressor," we measured plasma levels of ACTH and β-endorphin by radioimmunoassay (RIA). Two groups of 30 animals were anesthetized with an injection of Equithesin (0.2–0.3 ml/100 g i.p.), and an indwelling jugular cannula was placed according to the method described by Yoburn et al. (6). The animals were housed individually and allowed 72 h to recover from surgery. Both groups of animals were lightly anesthetized with ether and the stress group was wrap-restrained.

The indwelling jugular cannulas were used to draw 3 ml of blood from each animal. Each animal represented only one time point, and had blood drawn only one time. Blood was drawn 0, 10, 20, 30, and 60 min after stress or control ether exposure, in different groups of animals, into a 6-ml syringe containing 5 mg of ethylenediaminetetraacetic acid to prevent coagulation (n = 6 animals per time point). Blood samples were then centrifuged for 15 min at 20,000 rpm and 4°C, and the plasma was collected, divided into 0.5-ml aliquots, and frozen (−20°C) for assay at a later time. Radioimmunoassay kits for ACTH and β-endorphin were purchased from Immunonuclear Corporation. To establish baseline ACTH and β-endorphin levels, blood was drawn from 6 animals that had been neither wrap-restrained nor anesthetized with ether.

To study the change in hormone levels in stressed and nonstressed animals over a 24-h period, we followed a similar procedure. Blood was drawn 20 min after stress (the time of peak plasma ACTH concentrations), or after exposure to ether alone in the case of nonstressed animals. Adrenocorticotropic hormone and β-endorphin levels were measured in control and wrap-restrained rats at the same times of the day that transit had been assessed (1 AM, 4 AM, 7 AM, 10 AM, 1 PM, 4 PM, 5 PM, 7 PM, 10 PM).

Analgesia

Many types of physical and psychologic stress result in analgesia, a diminished response to a noxious stimulus. To evaluate the ability of wrap restraint to produce analgesia, rats were restrained, and sensitivity to a noxious heat stimulus was evaluated 10 min before, and 10, 20, 40, 60, and 90 min after the stress was applied. Control animals were tested 10 min before a brief ether exposure, as well as 10, 20, 40, 60, and 90 min afterwards. The analgesia test used was the tail flick assay (7), in which a beam of light is focused on the ventral side of the tail, and the latency to flick the tail away from the noxious stimulus is measured. A cutoff period of 15 s was used to prevent damage to the skin of the rat's tail. Three groups of 5 animals were used in the analgesia experiment. One group was anesthetized with ether and wrap-restrained. A second group was treated with the opioid antagonist naloxone (10 mg/kg s.c.) 10 min before wrap restraint. The third group served as a control group and were anesthetized with ether, but were not wrap restrained, and received saline subcutaneously. The percent analgesia was calculated according to the following equation:

$$\% \text{ Analgesia} = \frac{\text{Test latency} - \text{control latency}}{15 - \text{control latency}} \times 100.$$

Ulcers

We compared the ulcerogenic potential of wrap-restraint stress to classical cold restraint stress. Three groups of 5 animals per group were used. In the first group, animals were lightly anesthetized with ether and wrap restrained for 60 min. A second group was placed in wire mesh restrainers in a prone position for 60 min. The third group served as controls, and were neither anesthetized nor restrained in any fashion. After a 1-h exposure to restraint, the animals were killed and the stomach and proximal intestine were removed and cut open along the greater curvature, rinsed with saline, laid flat, and visually inspected for ulcers. Ulcers were scored according to the criteria described by Garrick et al. (8). Briefly, the total number of lesions was counted and the surface area damaged by individual lesions was measured by totaling the length of red streaks in millimeters for the entire glandular portion of each stomach. In addition, the feces were collected and counted, and evaluated as either formed and dry or watery and soft.

Hypophysectomy and Adrenalectomy

Hypophysectomized and adrenalectomized female Sprague–Dawley rats (150–200 g) and the appropriate sham surgeries were purchased from Harlan Breeders. Upon arrival, the adrenalectomized animals were given 2% saline drinking water and apple and orange segments, in addition to Purina Rat Chow, to improve their survival. Sham animals and hypophysectomized animals were also given apple and orange segments but normal drinking water. The animals were allowed 4–5 days to recover from shipping. Bilateral adrenalectomy was confirmed at the

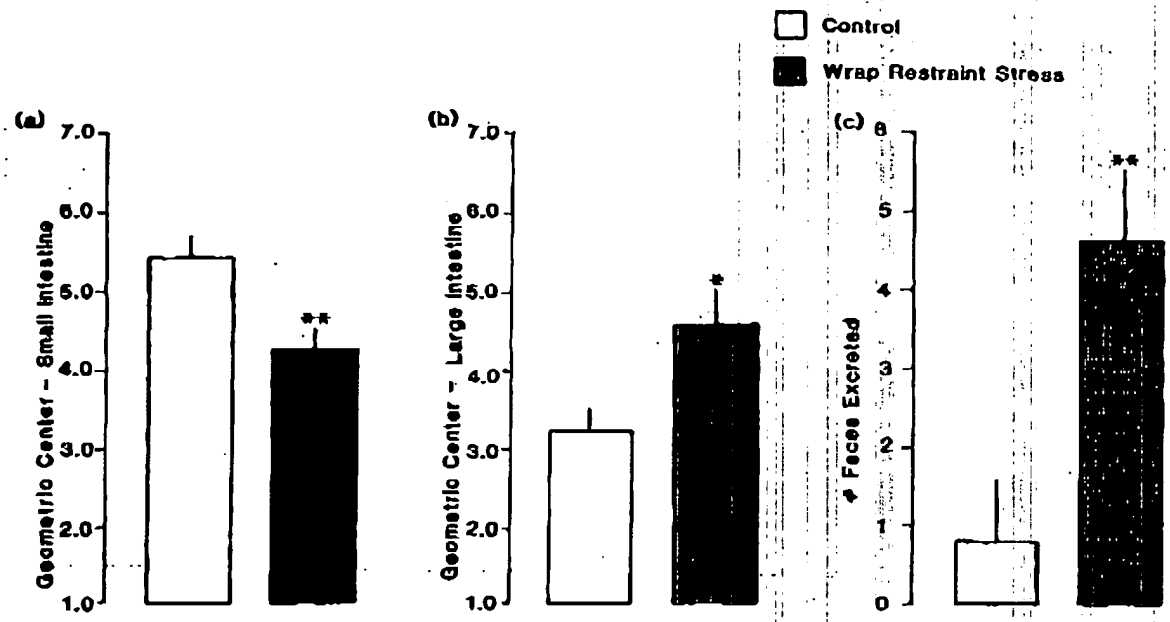

Figure 1. Effects of acute wrap-restraint stress on (a) small intestinal transit, (b) large intestinal transit, and (c) fecal excrement in rats. n = 5–7 animals per point. *$p \leq 0.05$, **$p \leq 0.01$ vs. control.

time of death by visual inspection for adrenal glands. The completeness of hypophysectomy was subjectively evaluated by scoring for the loss of guard hairs. Animals that retained guard hairs were not used in the study.

Statistics

The responses to wrap-restraint stress were tested for significant differences using Student's *t*-test, and for multiple comparisons using one- or two-way analysis of variance (ANOVA) followed by the Neuman–Keuls test for comparison of means (9).

Results

Gastrointestinal Effects

Wrap-restraint stress produced a significant alteration of small and large intestinal transit. The stress resulted in as much as 50% inhibition of transit along the small intestine. In contrast, restraint resulted in an increase in large intestinal transit, and an increase in fecal pellet output. Figure 1 shows the results of wrap-restraint stress on small and large intestinal transit. Panel *A* compares the geometric centers for small intestinal transit in control and wrap-restrained animals. The geometric center for the stressed group was significantly lower than control, indicating a net decrease in bulk flow through the small intestine. Panel *B* shows the geometric center of transit along the large intestine for control and stressed animals. Restraint stress resulted in an increase in large bowel transit, and an associated increase in fecal pellet output, the results of which are shown in panel C. All feces were formed and dry, and restraint stress did not result in diarrhea. Gastric emptying was not affected by wrap-restraint stress; gastric emptying was 55.6% ± 4.7% in control animals, compared with 54.3% ± 7.2% in wrap-restrained animals.

Ulcers

Figure 2 shows the absence of ulcers produced by wrap restraint compared with the ulcerogenic effects of cold restraint, a model classically used in the study of stress-induced ulcer formation. Animals were restrained by both methods for 60 min, at which time the stomachs were removed and the gastric and duodenal mucosa were inspected for erosions. No erosions developed after an hour of wrap-restraint stress. In contrast, cold restraint resulted in gastric erosion. The mean number of lesions was 17.5 ± 1.8 for the cold-restraint group. This suggests that cold restraint is a different type of perturbation, and may be a more severe form of stress than wrap restraint. Cold restraint also resulted in a greater degree of fecal excretion (11.3 ± 0.6 compared to 6.0 ± 0.8 for wrap restraint), and feces were soft or watery in half the animals exposed to cold restraint. Fecal excretion was increased in wrap-restrained animals compared with controls; however, all feces were formed and dry.

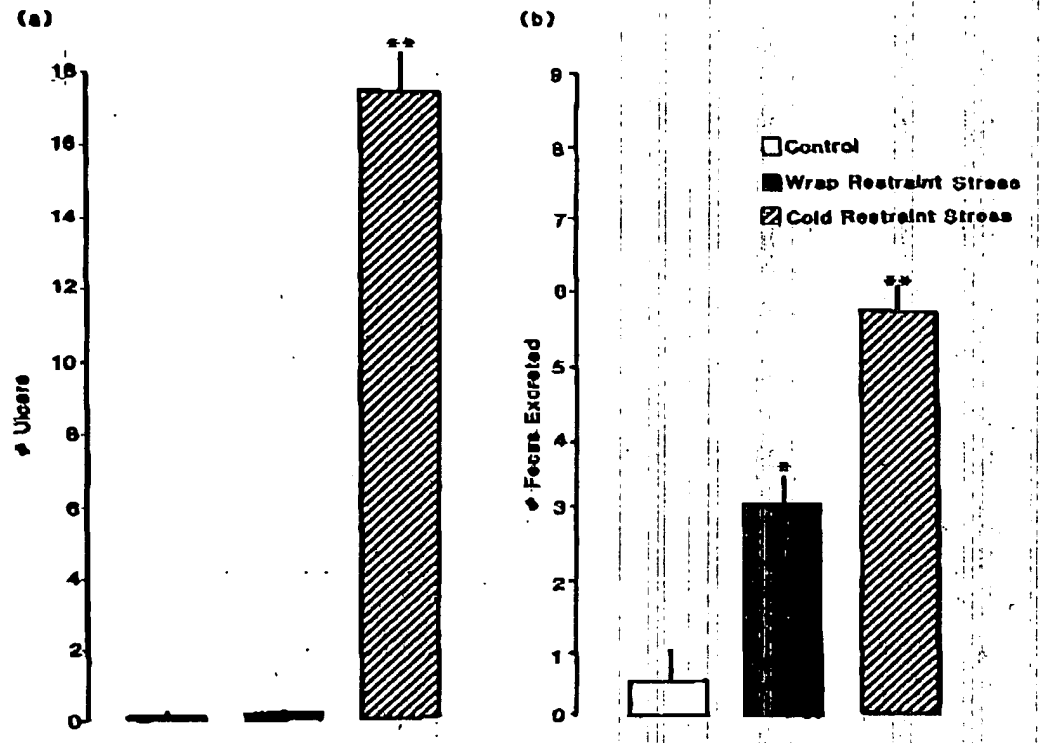

Figure 2. a. Formation of ulcers in response to 60 min of wrap-restraint and cold-restraint stress. *b.* The number of feces excreted in response to wrap-restraint and cold-restraint stress. n = 5 animals per group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ vs. control.

Adrenocorticotropic Hormone and β-Endorphin Release

To ascertain that the changes in transit induced by wrap restraint were associated with accepted markers of stress, plasma levels of ACTH and β-endorphin were measured by radioimmunoassay. Figure 3 shows the change in ACTH and β-endorphin levels in wrap-restrained and control animals over a 1-h period. Panel *a* shows that there was a rapid, eightfold increase in plasma ACTH levels within 20 min, and ACTH remained elevated for 30 min. Pituitary ACTH release is closely regulated by adrenal glucocorticoids. Release of glucocorticoids is stimulated by ACTH, and increasing concentrations of glucocorticoids inhibit the further release of ACTH. Adrenocorticotropic hormone levels returned to control values by 60 min. Exposure to ether is also a known stimulus of ACTH release (10), and there was a modest elevation in plasma ACTH levels of the control animals over time. However, the rise in ACTH in control animals was minimal compared with the levels generated by wrap-restraint stress. Panel *b* shows a similar release pattern for β-endorphin. Wrap-restraint stress resulted in a rapid increase in plasma β-endorphin concentrations. Peak concentrations occurred 20 min after stress was applied, and β-endorphin remained elevated for 60 min.

Analgesia

Acute wrap-restraint stress produced significant analgesia that lasted 10–20 min, as shown in Figure 4. The stress-induced analgesia was completely blocked by administration of the opioid antagonist naloxone (10 mg/kg s.c., 10 min before stress), suggesting that endogenous opioids may be involved in mediating the analgesic effects of wrap-restraint stress.

Circadian Effects

The effect of stress on small intestinal transit was strongly dependent on the time of day at which the experiment was performed. Figure 5 shows the effect of stress on small intestinal transit when the animals were wrap restrained at different times of the day. Radioactive chromium was administered after 25 min of stress, and transit was assessed 35 min later. Panel *a* compares the geometric centers for control and wrap-restrained animals at 1 AM, 4 AM, 7 AM, 10 AM, 1 PM, 4 PM, 5 PM, 7 PM, and 10 PM. The control geometric centers of transit remained fairly constant

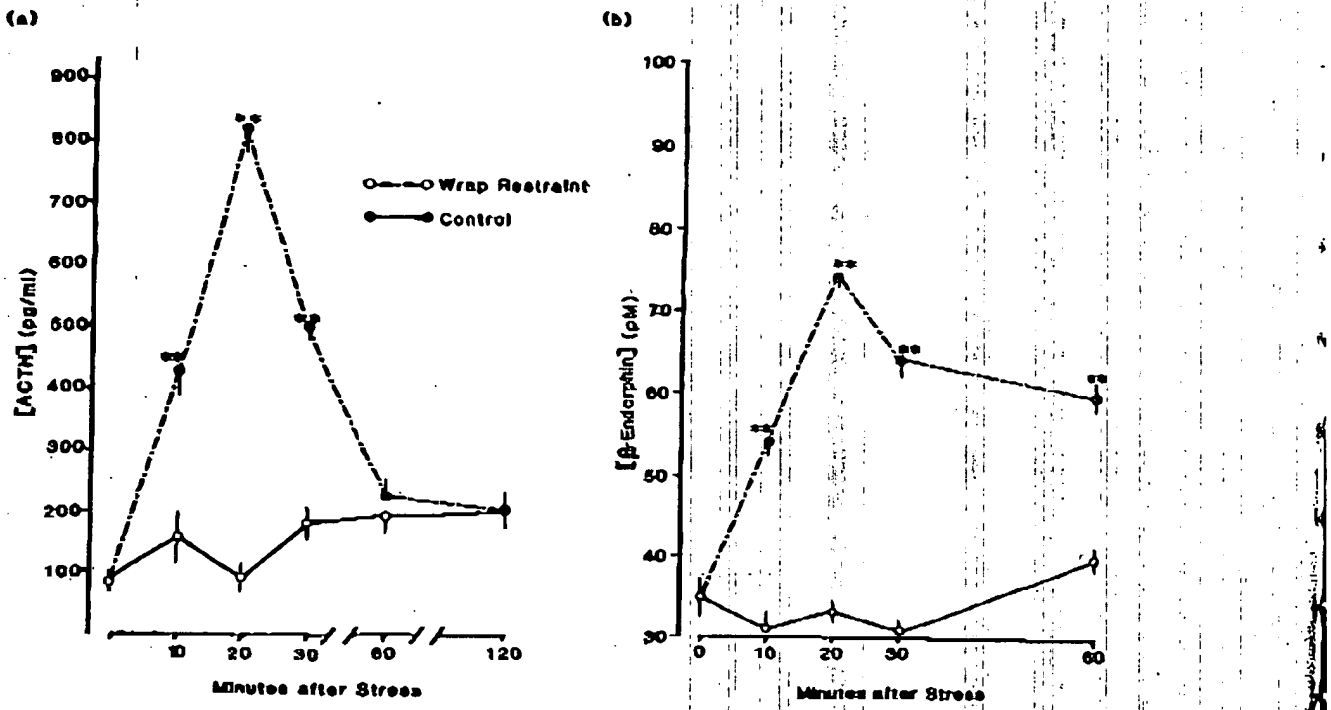

Figure 3. Wrap-restraint stress-induced (*a*) ACTH release and (*b*) β-endorphin release over time. Adrenocorticotropic hormone and β-endorphin plasma concentrations were determined by radioimmunoassay in samples taken from both control animals and stress animals. n = 3–4 animals per point. $^{**}p \leq 0.01$ vs. wrap-restrained animals.

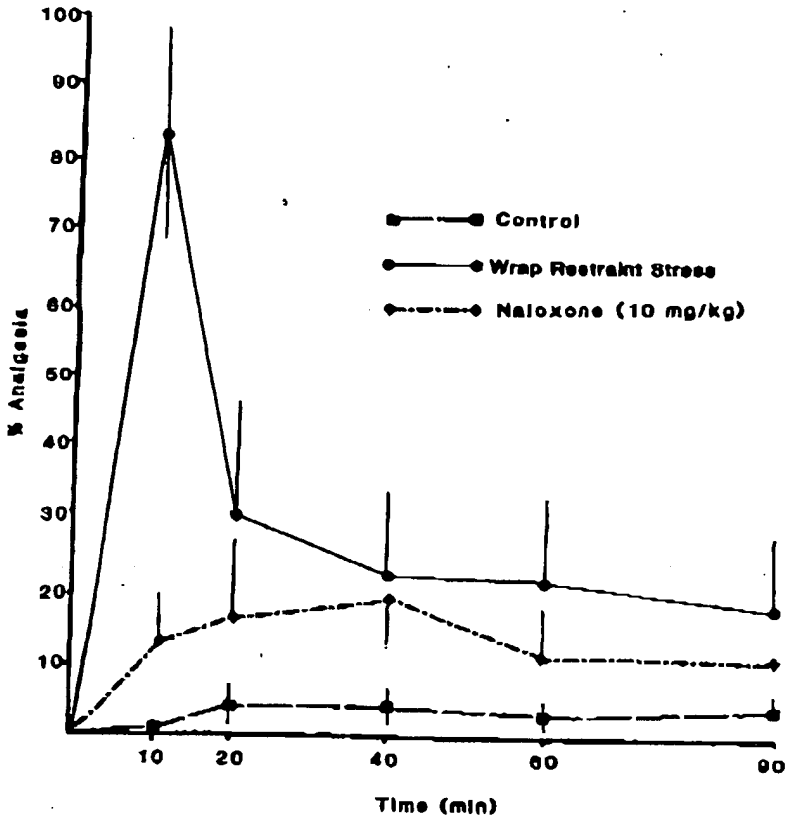

Figure 4. Percent analgesia expressed in control rats, stressed rats, and stressed rats treated with the opioid antagonist naloxone (10 mg/kg s.c., 10 min before stress). n = 5 animals per group.

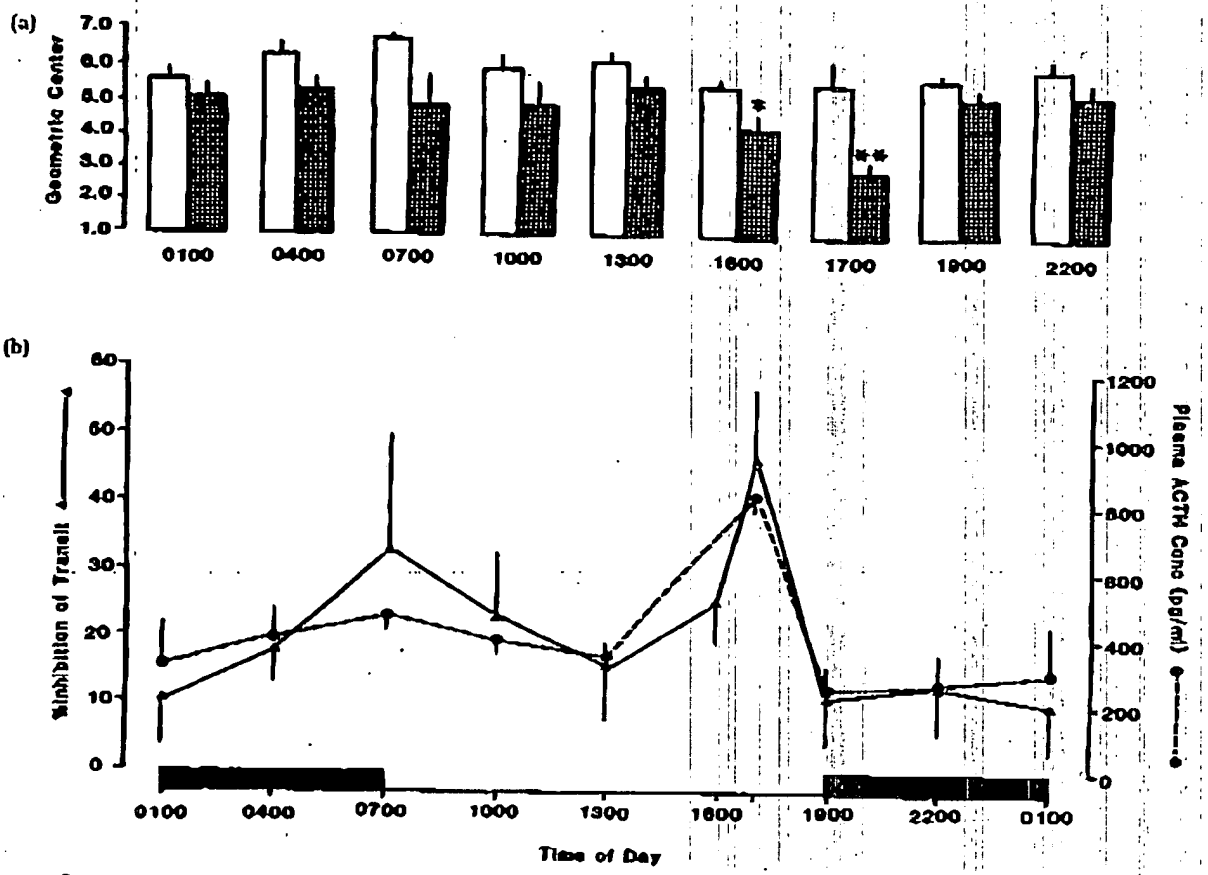

Figure 5. a. Geometric center of small intestinal transit for control animals (open bars) and wrap-restraint-stressed animals (hatched bars) over a 24-h period. n = 3–4 animals per point. b. A comparison of stress-induced inhibition of small intestinal transit (▲—▲) and stress-induced plasma ACTH concentrations (●—●) over a 24-h period. * $p \leq 0.05$, ** $p \leq 0.01$ vs. control.

over the 24-h period, ranging from 5.5 to 6.8. Transit along the small intestine of stressed animals was consistently slower than controls. However, the greatest differences between stressed and control groups occurred when the animals were tested in the late afternoon (4 PM and 5 PM). Only at these two time points did the differences between geometric centers for wrap-restrained and control animals become significant.

Adrenocorticotropic hormone release is known to follow a circadian pattern, and normal plasma levels are maximal in rodents just before the dark phase (i.e., late afternoon) (11). As the effect of stress on intestinal transit was found to be strongly influenced by the time of the day the experiments were performed, we compared the change in small bowel transit with changes in plasma ACTH concentrations associated with wrap-restraint stress at different times of the day. Figure 4B shows the relationship between stress-induced inhibition of small intestinal transit and stress-induced increased in plasma ACTH concentrations. Animals tested at 5 PM, immediately before the beginning of the dark phase, showed the greatest response in both stress-induced ACTH release and stress-induced intestinal dysfunction. At this time point, wrap restraint resulted in 46% inhibition of transit along the small intestine and a plasma ACTH concentration of 821.7 ± 38 pg/ml. A second, smaller peak in stress-induced inhibition of transit and release of ACTH occurred at the end of the dark phase, at 7 AM, but because of a high degree of variability, the results did not reach statistical significance. The similarity of the two responses to wrap-restraint stress was striking, and there was a strong correlation ($r = 0.95$) between the inhibition of small intestinal transit and stimulation of ACTH release over the entire 24-h period.

Adrenocorticotropic Hormone and β-Endorphin: Intestinal Effects

To determine the role of ACTH and β-endorphin as mediators of the effects of restraint on gastrointestinal function, we evaluated the ability of exogenously administered ACTH and β-endorphin to alter small and large intestinal transit. Table 1

Table 1. Intestinal Effects of Intravenous Adrenocorticotropic Hormone and β-Endorphin

	Dose ACTH (μg/kg i.v.)				
	0	0.1	0.3	1.0	3.0
SIT	5.19 ± 0.55	5.30 ± 0.33	4.80 ± 0.34	5.16 ± 0.84	4.75 ± 0.77
LIT	3.01 ± 0.37	3.33 ± 0.58	3.09 ± 0.08	2.94 ± 0.46	3.34 ± 0.59
	Dose β-endorphin (mg/kg i.v.)				
	0	0.1	0.3	1.0	
SIT	5.94 ± 0.34	4.24 ± 0.37	5.80 ± 0.24	6.21 ± 0.46	
LIT	3.44 ± 0.20	3.98 ± 0.47	3.02 ± 0.23	3.07 ± 0.56	

ACTH, adrenocorticotropic hormone; LIT, large intestinal transit; SIT, small intestinal transit. Effects of intravenously administered ACTH and β-endorphin on SIT and LIT in the rat. Data are expressed as the mean geometric center of transit ± SEM (n = 4–7 animals per point).

shows the results of these experiments. Neither ACTH nor β-endorphin affected intestinal transit when administered intravenously, even when the doses administered were much greater than the doses required to reach plasma concentrations of the hormones under stress conditions.

Hypophysectomy and Adrenalectomy

To evaluate the importance of the pituitary-adrenal axis in initiating intestinal abnormalities in response to stress, we studied the effects of wrap restraint on small intestinal transit in normal animals and animals in which the pituitary or adrenal glands were surgically ablated. Because these animals are so fragile, surgical implantation of intestinal cannulas was not possible, and small intestinal transit was evaluated after oral administration of the radioactive marker. The geometric center of small intestinal transit is similar in nonstressed animals when chromium is administered orally or is instilled directly into the proximal small intestine. It was not possible to study large intestinal transit in these animals. Neither hypophysectomy nor adrenalectomy affected stress-induced inhibition of small intestinal transit (Figure 6), indicating that the effects of acute stress on small intestinal transit are not mediated by pituitary or adrenally derived factors.

Discussion

The major finding of this study is that wrap restraint results in a significant alteration in intestinal transit. Small intestinal transit was inhibited, whereas large intestinal transit and fecal excretion were increased. The observation that a stimulus, wrap restraint, produced different responses in the small and large intestines suggests that different regions of the gastrointestinal tract are independently regulated and can react independently to the same perturbation.

The changes in intestinal transit induced by stress in the rat are similar to changes in intestinal motility induced by stress in humans. Functional bowel disease, including irritable bowel syndrome (IBS), is a common, often incapacitating, gastrointestinal disorder in humans manifested particularly at times of stress. Irritable bowel syndrome is a disorder, or group of disorders, that is diagnosed largely by symptoms; the etiology of the disorder is completely unknown. Because IBS may be a heterogeneous group of disorders, the symptomatology is varied in different patients, and problems of nomenclature and classification have led to controversy in the field. However, our findings are supported by a number of reports. For example, psychologic stress results in a suppression of normal fasting migrating motor complex motility patterns in the proximal small intestine (12), which is exaggerated in patients with IBS (13). A physical stress, immersion of the hand in cold water, has been reported to delay orocecal transit as measured by the breath hydrogen test (14), and both physical and psychologic stressors have been reported to inhibit small intestinal transit in patients with IBS (15). Stress reportedly increases colonic motility in both normal subjects and subjects with IBS (16,17). However, myoelectric slow waves or electrical control activity of the colon are reportedly not affected by stress (18). Diarrhea is a common symptom of stress, and Barone and coworkers (19) have shown that cold-restraint stress increases fecal pellet output and colonic transit in rats. When wrap-restraint stress is the type of stimulus employed, the resultant changes in gastrointestinal motor function, i.e., decreased small intestinal transit, increased large intestinal transit, and increased fecal output, are very similar to symptoms reported in humans in response to stress, or symptoms associated with functional bowel disease thought to be induced or exacerbated by stress. This indicates that wrap-restraint stress, a mild, nonul-

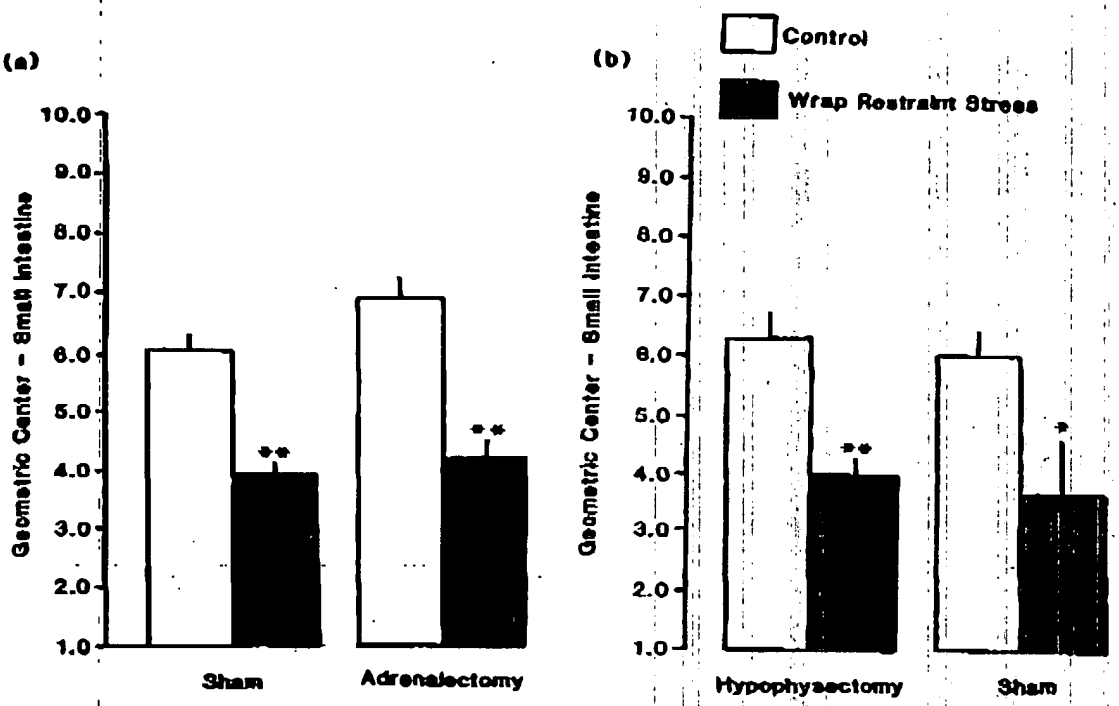

Figure 6. Effects of surgical ablation of the adrenal gland and the pituitary on wrap-restraint stress-induced inhibition of small intestinal transit. *a*. The geometric center for small intestinal transit in adrenalectomized and sham-adrenalectomized animals in control and stress conditions. *b*. The geometric center for small intestinal transit in hypophysectomized and sham-hypophysectomized animals in control and stress conditions. n = 5 animals per point. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ vs. control.

cerogenic model of stress, may be a useful model in which to study the effects of stress on the intestine.

Wrap restraint is a true stress stimulus, as indicated by ACTH and β-endorphin release and the production of analgesia. Both endocrine changes and analgesia are hallmark responses to stress. We found that the degree of intestinal dysfunction produced by stress was strongly dependent on the time of the day that the animals were stressed, suggesting a circadian influence on stress-induced intestinal dysfunction. Puglisis-Allegra et al. (20) have reported a similar circadian influence on stress-induced analgesia in mice. In addition, the gastrointestinal tract itself exhibits definite circadian patterns in secretory and motor functions that persist in fasted animals, suggesting that the patterns are not due to feeding cycles (21–23), and a circadian variability has been reported in the susceptibility of the gastric mucosa to aspirin-induced lesions (24). A close correlation occurred in our studies between stress-induced inhibition of small intestinal transit and stress-induced ACTH release. Rats were most sensitive to the effects of stress in the late afternoon, 4–5 PM, at a time when circadian variations in plasma levels of ACTH are maximum, and when stress-induced release of endocrine factors is most exaggerated. Interestingly, Kumar et al. (25,26) have reported that symptoms are most severe for IBS patients in the early morning (the equivalent of late afternoon in rats and other nocturnal species), and they suggest a circadian influence on the sensitivity of the human bowel to stress.

The observation that stress-induced changes in ACTH levels and intestinal transit occur in parallel suggests that endocrine messengers may play a role in mediating the effects of stress on the gut. The hypothalamic-pituitary-adrenal axis is the endocrine system most central to the stress response (for review, see Reference 27), and ACTH and β-endorphin are released concomitantly from the pituitary in response to stress (28). However, exogenously administered ACTH and β-endorphin were completely ineffective in altering intestinal transit, even when the doses administered were 100 times greater than those required to reproduce plasma concentrations of the hormones under conditions of stress (30 ng/kg ACTH i.v. results in a plasma concentration of 540 ± 38 pg/ml within 5 min, which is equivalent to stress-induced plasma concentrations). Furthermore, surgical ablation of the pituitary and adrenal glands did not affect the response of the small intestine to stress. Therefore, stress-induced changes in gastrointestinal transit are not mediated by pituitary or adrenally derived factors. Parallel changes in

stress-induced endocrine function and intestinal dysfunction are highly correlated, but are not causally related.

A close correlation between stress-induced ACTH release and stress-induced changes in intestinal function may indicate that both the endocrine and intestinal responses to stress are initiated by a common regulator. Corticotropin-releasing factor is the major hypothalamic peptide released in response to stress. The endocrine activities of corticotropin-releasing factor have been well characterized. In addition to its specific endocrine functions, corticotropin-releasing factor also produces changes in behavior, cardiovascular function, metabolic function, and central nervous system activity that are indicative of stress (29). This suggests that corticotropin-releasing factor may be a "master regulator" of both endocrine and neural responses to stress. It is interesting to speculate that this hypothalamic peptide may also be involved in regulating intestinal responses to stress.

In conclusion, wrap-restraint stress resulted in altered small and large intestinal transit. The divergence in the effects of stress on the small intestine (which is inhibited) and the large intestine (which is stimulated) emphasizes the independent regulation that exists in different regions of the gastrointestinal tract. Wrap-restraint stress is a mild, nonulcerogenic stressor that reproduces the symptoms associated with stress-related intestinal dysfunction in humans, suggesting that it may be an appropriate model in which to study the effects of stress on the gut.

Wrap-restraint stress-induced intestinal dysfunction was strongly dependent on the time of day at which the experiments were performed, suggesting a circadian influence. A strong correlation between stress-induced ACTH release and stress-induced intestinal dysfunction might have implied a causal relationship between ACTH or other endocrine messengers and the response of the gut to stress; however, neither exogenously administered ACTH nor β-endorphin affected intestinal function, and ablation of the pituitary and adrenal glands did not affect stress-induced intestinal dysfunction. Thus, neither pituitary nor adrenally derived factors are involved in mediating the effects of stress on the gut. The mechanisms by which stress alters intestinal function remain unknown.

Note added in proof. Since submission of this manuscript, evidence for a critical role for corticotropin-releasing factor in colonic effects of stress has been presented (Williams CL, Peterson JM, Villar RG, Burks TF. Corticotropin-releasing factor directly mediates colonic responses to stress. Am J Physiol 1987;253:G582–6).

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